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13. ABSTRACT (Maximum 200 Words)

Intestinal bacteria are involved in estrogen metabolism. Thus, inter-individual differences in intestinal bacterial populations may contribute to variation breast cancer risk via effects on estrogen metabolism. One-third to one-half of the population harbors the yet-to-be-identified bacteria that metabolize daidzein, a soy isoflavone, to equol. Studies suggest that equol production may be associated with reduced breast cancer risk, but mechanisms for this relationship are unknown. Our objective was to determine whether fecal bacterial metabolism of estrogens differs according to equol-producer phenotype. A method was developed for the extraction and gas chromatography-mass spectrometry quantification of estrogens from fecal incubations. Previously frozen fecal samples from female equol producers (n=20) and non-producers (n=13) were incubated anaerobically in media for 5d at 37°C with the following estrogens: estrone, estradiol, estriol, 2-hydroxyestrone, 16α-hydroxyestrone, and 2-methoxyestrone. Fecal bacteria from equol-producers were more likely than non-producers to convert 2-hydroxyestrone to 2-hydroxyestradiol. Furthermore, equol-producers, compared to non-producers, converted significantly more estrone to estradiol, and 16α-hydroxyestrone to estriol. These findings suggest differences may exist between equol-producers and non-producers in bacterial estrogen metabolism. However, the conversion of 16α-hydroxyestrone to estriol, and the conversion of estrone to estradiol could represent potentially beneficial and detrimental, respectively, pathways in terms of breast cancer risk.

14. SUBJECT TERMS
Equol, estrogen metabolism, bacteria, isoflavone, daidzein, in vitro incubation

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INTRODUCTION

Hormonal exposure is associated with risk of breast cancer and determinants of circulating levels of estrogens and of estrogen metabolism are among the primary risk factors for breast cancer (1). Estrogens are metabolized by various species of intestinal bacteria, and certain reactions may be species, or even strain, specific. Thus, inter-individual differences in intestinal bacterial populations may contribute to variation in disease susceptibility through effects on the metabolism and subsequent exposure to estrogens. The equol-producer phenotype is a marker of inter-individual differences in gut bacterial populations. Daidzein, a soy isoflavone, is metabolized by colonic microflora to equol, which can be measured in blood and urine. However, only one-third to one-half of the population harbors the yet-to-be-identified bacteria that produce equol (2). Studies suggest that equol production may be associated with a lowered risk of breast cancer (3, 4), and this is potentially via effects of the intestinal bacteria responsible for, or associated with, equol production on circulating levels of hormones. Our objective is to determine, for the first time and in an efficient and non-invasive manner, whether estrogen metabolism by human fecal bacteria differs according to equol-producer phenotype. Fecal samples from female equol producers and non-producers will be incubated with selected estrogens under anaerobic conditions at 37°C. Existing protocols for the extraction and gas chromatography-mass spectrometry (GC-MS) quantification of estrogens and their metabolites from urine will be adapted for their extraction and quantification from brain heart infusion (BHI) media inoculated with feces. The *in vitro* metabolism of estrogens will be compared between equol-producers and non-producers. If the colonic bacteria responsible for, or associated with, the equol-producer phenotype are involved in hormone metabolism, the equol-producer phenotype could have a bearing on the concentrations of some circulating estrogens and thereby ultimately impact breast cancer risk. Data generated from this study could provide valuable information regarding potential mechanisms for the relationship between equol production and breast cancer risk, and could support the rationale for more extensive study to test hypotheses related to risk of breast cancer in equol-producers versus non-producers.

BODY

The tasks outlined in the Statement of Work have been completed, and a manuscript is in preparation. The key research accomplishments associated with each task are summarized below.

Task 1

To develop protocols for the extraction and gas chromatography-mass spectrometry (GC-MS) analysis of target estrogens from fecal incubations, Months 1-4:

a. Adapt established protocols for the extraction and quantification of target estrogens from urine to their extraction and quantification from fecal incubations.

The development of a method for the extraction and quantification of estrogens from fecal incubations was based on previous assays for the extraction and quantification of estrogens and isoflavones (plant estrogens) from urine. A suitable GC-MS column (DB-17MS; 30m x 0.25 mm x 0.25 μm) was purchased and conditioned for use. Pure standard solutions of estrogens in methanol were injected onto the column to select target ions and to derive initial instrument parameters including retention times and number of trimethylsilyl sites (derivatization sites).

a.i. Establishing stability data on target estrogens

Initial experiments were conducted by adding known concentrations of estrogens to 5 ml brain heart infusion (BHI) media and extracting estrogens from a 1 ml aliquot of this solution. However, recoveries of estrogens were erratic. When known concentrations of estrogens were added to 1 ml media or DI water and the entire aliquot was extracted, recoveries were found to be consistent. Therefore, 1 ml media was used as the starting volume for all experiments with estrogens and the entire aliquot was extracted.

Several experiments were conducted to determine the stability of the pure estrogen standards, including assessing estrogen levels [estrone (E1) and 17β-estradiol (E2)] on days 0, 1, 2, 3, 4, and 5 of an incubation, and extracting estrogens [E1, E2, 2-methoxyestrone (2-methoxy E1), 2-hydroxyestrone (2-OH E1), 16α-hydroxyestrone (16α-OH E1), estriol (E3), 16 keto-estradiol (16-keto E2), and 16 epi-estriol (16-epi E3)] that had been spiked into BHI media with or without feces, and from deionized (DI) water; samples were extracted either immediately after preparation, or following 24 hours at –20°C. Recoveries of estrogens were acceptable and consistent, with the exception of 2-OH E1; recoveries of 2-OH E1 were erratic and ranged from 0 to 100%. Other investigators (5) similarly have reported problems with the stability of 2-OH E1.

a.ii. Optimizing extraction method and target estrogen quantification parameters

<u>*Extraction solvent:*</u> Preliminary extractions of estrogens spiked into DI water, BHI media, and BHI media that had previously been incubated with feces for 5 days at 37°C, were carried out using diethyl ether. Recoveries of the internal standard were consistent, but recoveries of some target estrogens, including E3, 16-keto E3, and 16-epi E3, were low and variable. Hexane, acetonitrile, and ethyl acetate were subsequently tested as extraction solvents, and ethyl acetate gave the best calibration curves for estrogen standards and good recoveries for spiked samples. Additional studies with spiked DI water and media were completed with varying amounts of internal standard and target concentration ranges, and all gave satisfactory recoveries.

In order to minimize variability introduced by the extraction of estrogens from media, all standards used in calibration curves for experiments conducted as part of Task 2 were extracted from BHI media.

The steps involved in the extraction and GC-MS analysis of estrogens and isoflavones are shown in appendix 1 (figure 1). Isoflavones [daidzein, equol, O-desmethylangolensin (O-DMA), and dihydrodaidzein (DHD)] also were measured in the same GC-MS run as the estrogens, in order to assess the *in vitro* metabolism of daidzein by fecal bacteria from the equol-producers and non-producers.

Internal standard: Several internal standards were considered for use. Ethinyl estradiol and beclomethasone were considered initially, but interference with other target estrogens and instability, respectively, made them unsuitable. Enterodiol and deuterated estrone were found to be suitable, as recoveries were consistently good. Enterodiol was adopted as the internal standard.

a.iii. Establishing instrument and method detection limits for target estrogens

Instrument and method detection limits were established by running at least seven each of spiked BHI media and DI water samples per day over a period of three non-consecutive days. The concentrations of estrogens within each matrix were 5 ng, 10 ng, and 15 ng ('on column' values). The instrument detection limit was initially determined to be 2 ng (on column) for 17α-E2, 2-OH E2, 2-methoxy E2, E1, 2-OH E1, 16α-OH E1, E2, and 2-methoxy E1, but not 17-epi E3, E3, 16-epi E3, and 16-keto E3. The lowest concentration that all analytes were consistently detected was 10 ng (on column).

a.iv. Method validation (accuracy, precision, limit of quantitation, specificity, linearity, range, ruggedness, robustness)

Accuracy: the percent of each analyte recovered from spiked DI water and spiked BHI media was assessed. Three concentration levels (30 ng, 50 ng, and 70 ng on column) were considered for each analyte in each matrix. With the exception of 2-OH E1, E3, and 16-keto E2 (all at 30 ng only), recoveries were at, or above, 90% for all analytes for both matrices.

Precision: the degree of repeatability was measured by comparing the results of five spiked DI water and BHI media (at a concentration of 70 ng on column) measurements over a period of 5 days. Intra-run coefficients of variation (CVs) for all analytes were at, or less than, 5% for both matrices. The inter-run CVs for all analytes were less than 15%, except for 16α-OH E1 and 16-keto E2, which had CV's at or below 20%.

Limit of quantitation: the lowest concentration of all analytes that could be determined with acceptable precision (less than or equal to 20 % CV for inter- and intra- run CVs) and acceptable accuracy (at least 80% recovery) was 15 ng (on column). However, data on estrogen levels between 2 and 15 ng (on column) were considered semi-quantitative, and were used in analyses comparing estrogen metabolism between equol-producers and non-producers.

Specificity: identification of each analyte was made based on retention time, peak shape, and the percent recovery of up to three secondary ions for each target; thus, the specificity for this GC-MS (single ion monitoring mode) method was determined to be excellent. Furthermore, the primary ions (upon which quantification is determined) were different for each target analyte, and intra- and inter- run % CVs for retention times were each less than 1%.

Linearity: seven or more calibration standards were prepared in BHI media and extracted and analyzed with each batch of samples. The linear coefficients for each analyte were generally 0.99 or

better, with at least 6 concentration levels. Occasionally, however, the calibration curves for 16-epi E3, 16-keto E2, and 16α-OH E1 were 0.98 with five concentration levels.

Range: the established range of recovery for all analytes was determined to be 10 ng to 100 ng (on column).

Ruggedness: variability in results generated by different instruments, analysts, labs etc. (i.e., the method ruggedness) was not determined in this study because one technician analyzed all samples using the same instrument.

Robustness: the method was proven to be fairly robust. Spiked DI water and spiked BHI media were subjected to different pH ranges (pH 3, 4, and 6), temperatures (room temperature versus –20°C) and GC parameters (slightly different oven and injector temperatures and carrier gas flows). These deliberate variations in method parameters did not significantly change results. All were within the acceptable limits of accuracy and precision stated earlier. However, the recovery for 2-OH E1 was variable, which has been noted by others (5).

Task 2
To carry out an extensive investigation of hormone metabolism by fecal bacteria, Months 5-12:

a. A series of *in vitro* incubations will be carried out to investigate the metabolism of target estrogens by fecal bacteria from non-identifiable equol-producers (n=21) and non-producers (n=13). The metabolism will be compared and contrasted between equol producers and non-producers.
Equol-producer status: Equol-producer status associated with each fecal sample had been determined prior to this study using a three-day soy challenge (6, 7). Fecal samples from 34 individuals were used; 20 were from equol-producers, 12 were from equol non-producers, and 2 were from individuals who had trace amounts of equol in their urine sample (i.e., where a definitive classification of equol-producer status could not be made based on their urine sample; these samples had been incorrectly assigned as an equol-producer and an equol non-producer in the approved Statement of Work).

Experimental methods: For each incubation run, an aliquot of thawed feces (approximately 1-2 ml of a slurry of feces in 10% glycerol) was added to 5 ml autoclaved BHI media [BHI media had been supplemented with vitamin K1 (0.05 mg / 100 ml), heme (0.5 mg / 100 ml), and L-cystine (50 mg / 100 ml)] and inverted several times to mix. 20 μl of this bacterial broth was added to 1 ml BHI media containing 14 μg of an estrogen. In addition, 100 μl of the bacterial broth was added to 5 ml BHI media containing 50 μg daidzein. All estrogen incubations were carried out in triplicate, and daidzein incubations in duplicate. Samples were incubated under anaerobic conditions for 5 days at 37°C with the following estrogens: estrone (E1), estradiol (E2), estriol (E3), 2-hydroxyestrone (2-OH E1), 16α-hydroxyestrone (16α-OH E1), and 2-methoxyestrone (2-methoxy E1). E3 was removed from the target list after 16 incubations (8 equol-producers and 8 non-producers) because there was no metabolism of E3 by fecal bacteria; others (8) have reported similar findings. The following estrogens were included in the calibration curves for quantifying estrogens and their metabolites after the 5-day incubation: E1, E2, E3, 2-OH E1, 16α-OH E1, 2-methoxy E1, 16-keto E2, 16-epi E3, 17α-E2, 2-OH E2, 2-methoxy E2, and 17-epi E3.

Control tubes: Three control tubes per estrogen were prepared for each incubation run; 1 ml BHI media plus estrogen which was frozen immediately (i.e., day 0 tubes), 1 ml BHI media plus estrogen and feces which was frozen immediately (i.e., day 0 tubes with feces), and 1 ml BHI media plus estrogen that was incubated for 5 days at 37°C. In addition, a tube containing media only was incubated (to verify that the media had not become contaminated during preparation), and, in the majority of runs, a tube containing daidzein without feces also was incubated (to monitor for losses of daidzein). After the 5-day incubation, all tubes containing fecal inoculate showed visible signs of bacterial growth.

Data analysis: All estrogens were incubated with feces in triplicate (see appendix 1; figure 1). Some sets of triplicates showed conversion to a particular metabolite in all tubes, in two tubes, or in one tube only. Chi square analysis was used to determine differences between equol-producers and non-producers in the number of individuals whose fecal bacteria produced a particular metabolite; if any of the tubes in the triplicate showed conversion to a particular metabolite, the individual was classed as a converter. To determine differences between equol-producers and non-producers in the percent conversion of an estrogen to a metabolite, two analyses were conducted: a) using data from only the individuals and tubes that showed conversion, i.e., if two out of the three tubes produced a

specific metabolite, the mean of the two tubes was taken, and if only one out of the three tubes produced a specific metabolite, the percent conversion was based on data from that one tube; b) using data from all individuals and tubes, i.e., averages were calculated for all tubes irrespective of whether or not conversion had occurred in all tubes.

<u>Daidzein metabolism:</u> There were no losses of daidzein following incubation for 5 days at 37°C without feces. Fecal bacteria from one individual, who had been classed as an equol-producer based on their urine sample, did not convert daidzein to equol *in vitro*; thus, their data are not included in the results described below. When daidzein was incubated with feces from the remaining 19 individuals classed as equol-producers according to their urine sample and 1 of the individuals who had trace amounts of equol in their urine sample, equol was produced; of these, 15 produced equol only and 5 produced equol and DHD. When daidzein was incubated with feces from 12 individuals classed as equol non-producers according to their urine sample and 1 of the individuals who had trace amounts of equol in their urine sample, no equol was produced; of these, 4 did not metabolize daidzein, 4 produced DHD, 3 produced O-DMA, and, 2 produced DHD and O-DMA. The findings reported below are based on data from 20 equol-producers and 13 equol non-producers.

<u>Recovery of estrogens from control tubes and test samples:</u> Recoveries of target estrogens from the three control tubes were excellent [≥93%; appendix 2 (figure 2), panel A] with the exception of 2-OH E1; recoveries of 2-OH E1 from control tubes averaged approximately 30% [appendix 2 (figure 2), panel A). Total recoveries of estrogens and their metabolites after a 5-day incubation with feces were excellent for E1, E2, and E3 (≥88%). Recoveries of 16α-OH E1 and its metabolites from samples incubated with feces averaged 87% and 65% for equol-producers and non-producers, respectively, and recoveries of 2-methoxy E1 and its metabolites averaged 70% and 63% for equol-producers and non-producers, respectively [appendix 2 (figure 2), panel B]. These recoveries are lower than were observed with control tubes (≥93% and ≥98% for 16α-OH E1 and 2-methoxy E1, respectively), suggesting that there may have been production of some unidentified metabolites. Recovery of 2-OH E1 plus its metabolites was highly variable for incubated samples, and averaged less than 40% following the 5-day incubation [appendix 2 (figure 2), panel B]. Other investigators have reported similar problems with 2-OH E1 (5).

<u>Metabolism of estrogens among all individuals:</u> There was no metabolism of E3 by fecal bacteria from equol-producers (n=8) or non-producers (n=8). Substantial inter-individual variation in the metabolism of all other estrogens was apparent, as evidenced by the range of percent conversions shown in table 1 (appendix 3). The metabolism of estrogens in our study is in agreement with other in vitro studies using human fecal bacteria. For example, Järvenpää et al. (8) and Lombardi et al. (9) reported the inter-conversion of E1 and E2, and the metabolism of 16α-OH E1 to E3. Järvenpää et al. also reported no metabolism of E3 by intestinal bacteria (8), and the conversion of 2-OH E1 to 2-OH E2, 2-methoxy E1 to 2-OH E1, and 2-methoxy E1 to 2-OH E2 (5).

<u>Metabolism of estrogens among equol-producers and non-producers:</u> A summary of the *in vitro* metabolism of estrogens by fecal bacteria from equol-producers and non-producers is shown in table 1 (appendix 3). Significantly more equol-producers than non-producers metabolized 2-OH E1 to 2-OH E2 (χ^2=4.07, p=0.04). In addition, equol-producers, compared to non-producers, converted significantly more E1 to E2, and significantly more 16α-OH E1 to E3; these findings were the same when considering only those that showed conversion or all samples. All other differences between equol-producers and non-producers were not statistically significant (p>0.05). Some metabolites

were produced only by equol-producers (i.e., the production of 2-methoxy E2 from 2-OH E1 and E2 from 2-methoxy E1) or by equol non-producers (i.e., the production of 10-keto E2 from 16α-OH E1). However, it is possible that the sample size in this study was too small to determine whether or not this was due to equol-producer phenotype.

b. A final report and manuscript will be prepared.
A manuscript using data generated from this study is currently in preparation (appendix 4) and will be submitted to the Journal of Steroid Biochemistry and Molecular Biology for publication.

c. Depending on the findings of the study, an application for funds to carry out a more extensive evaluation of differences between equol-producers and non-producers in relation to circulating hormones and other hormone dependent factors related to risk of breast cancer will be prepared.
Of the six estrogens that were examined within this *in vitro* system, a significant difference between equol-producers and non-producers was observed for the metabolism of three estrogens, 2-OH E1, E1, and 16α-OH E1. However, problems with the measurement and / or stability of 2-OH E1 limited the interpretation of the findings in relation to the bacterial metabolism of 2-OH E1. It would be interesting to conduct further studies to evaluate potential differences between equol-producers and non-producers in the metabolism of other estrogens, and androgens, given that E1, E2, and E3 are derived from androgenic precursors, and that significant differences between equol-producers and non-producers in circulating levels of androgens also have been reported (4).

KEY RESEARCH ACCOMPLISHMENTS

- A method was developed for the extraction and GC-MS quantification of estrogens from BHI media after incubation with human feces

- For the first time, the *in vitro* metabolism of estrogens by fecal bacteria from equol-producers and non-producers was compared, and it was observed that:
    - There was considerable inter-individual variation in estrogen metabolism, irrespective of equol-producer status
    - Significantly more equol-producers than non-producers metabolized 2-OH E1 to 2-OH E2
    - Fecal bacteria from equol-producers, compared to fecal bacteria from equol non-producers, metabolized significantly more E1 to E2
    - Fecal bacteria from equol-producers, compared to fecal bacteria from equol non-producers, metabolized significantly more 16α-OH E1 to E3

REPORTABLE OUTCOMES

An abstract will be prepared for submission to the American Association for Cancer Research Annual Meeting, 2005, and to the Era of Hope 2005 Department of Defense Breast Cancer Research Program meeting.

A manuscript is currently in preparation (appendix 4), and will be submitted to the Journal of Steroid Biochemistry and Molecular Biology for publication.

LIST OF PERSONNEL RECEIVING PAY FROM THE RESEARCH EFFORT

Charlotte Atkinson, PhD
Wendy Thomas, BS
Meredith Hullar, PhD

CONCLUSIONS

We examined, for the first time, the *in vitro* metabolism of estrogens by fecal bacteria from equol-producers and non-producers. Data from some studies (3, 4) had suggested that equol-producers were at a lowered risk of hormone-dependent cancers including breast cancer, but no studies had assessed potential mechanisms for such a relationship. We hypothesized that the intestinal bacteria responsible for, or associated with, equol production are involved in hormone metabolism. Our *in vitro* incubations of human feces from known equol-producers and non-producers with selected estrogens showed that a significantly greater proportion of equol-producers metabolized 2-OH E1 to 2-OH E2 than equol non-producers. In addition, equol-producers, compared to non-producers, metabolized significantly more E1 to E2 and 16α-OH E1 to E3. If these differences exist *in vivo*, they may ultimately have an impact on breast cancer risk. However, the metabolism of 16α-OH E1 to E3 represents the metabolism of an estrogenic, and potentially genotoxic (10), estrogen to a less potent estrogen (11), whereas the metabolism of E1 to E2 represents the metabolism of a less potent estrogen to a more potent estrogen (12). Thus, although our findings suggest that there may be differences between equol-producers and non-producers in the metabolism of estrogens, they do not provide definitive data on a mechanism for the reported associations between the ability to produce equol and breast cancer risk (3, 4).

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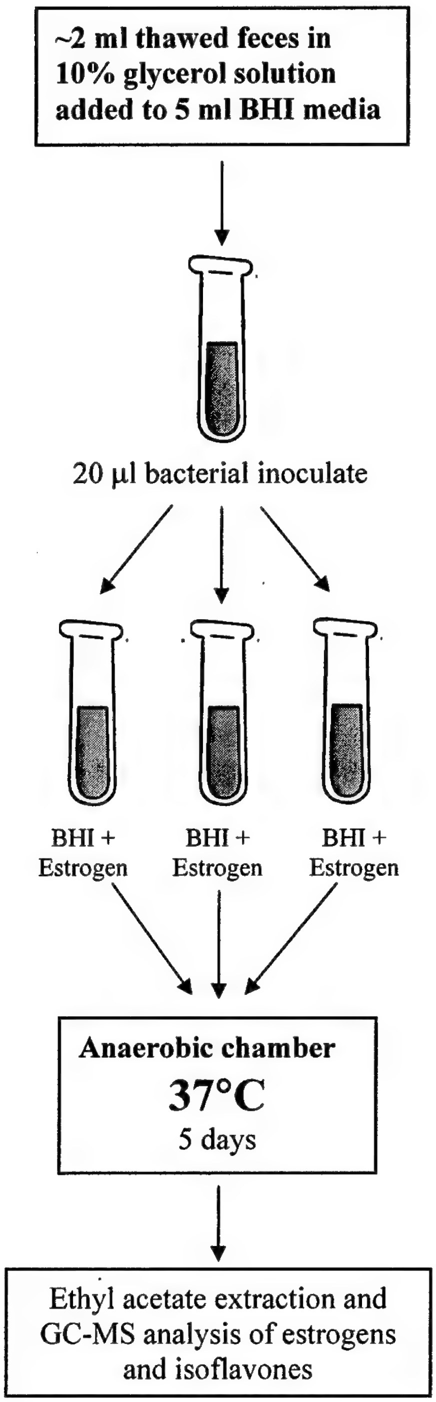
APPENDICES

1. Figure 1
2. Figure 2
3. Table 1
4. DRAFT manuscript

Appendix 1

Figure 1. Outline of experimental procedures

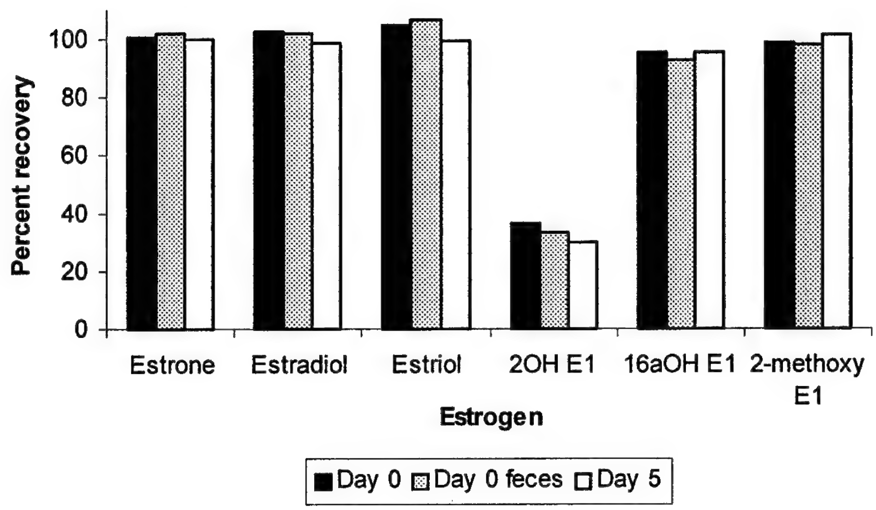


- Autoclaved BHI media (3.7 g / 100 ml DI water) was supplemented per 100 ml with vitamin K (0.05 mg), heme (0.5 mg), and L-cystine (50 mg).
- ~2 ml thawed feces in 10% glycerol solution was added to 5 ml BHI media; 20 μl of this bacterial broth was added to 1 ml BHI media containing 14 μg / ml estrogen in triplicate. In addition, 100 μl of this bacterial broth was added to 5 ml BHI media containing 10 μg / ml daidzein in duplicate.
- Screw caps were loosely screwed onto each tube, and tubes were incubated at 37°C for 5 days.
- After incubation, 100 μl enterodiol (50 ng / μl; internal standard) and 2 ml ethyl acetate was added to each 1 ml sample (for the daidzein tubes, 1 ml was used for extraction).
- Tubes were vortexed for 2 minutes, centrifuged for 20 minutes at 3200 rpm, then the ethyl acetate layer was removed into a clean test tube.
- The ethyl acetate extraction was repeated twice more, and all three ethyl acetate layers were combined in the same tube.
- Samples were evaporated to dryness under nitrogen at 37°C, and, if not derivatized immediately, re-hydrated with 0.5 ml methanol and stored at -20°C. Prior to derivatization, stored samples were brought to room temperature and evaporated to dryness under nitrogen at 37°C.
- For derivatization, 200 μl 15% MSTFA + TMCS solution (Pierce Chemical Co.; Rockford, IL) was added to each sample, and samples were incubated at room temperature for 30 minutes to produce trimethylsilyl (TMS) derivatives.
- Estrogens (and isoflavones) were quantified using an HP 5973 MSD GC-MS with Chemstation software (Agilent Technologies; Palo Alto, CA).

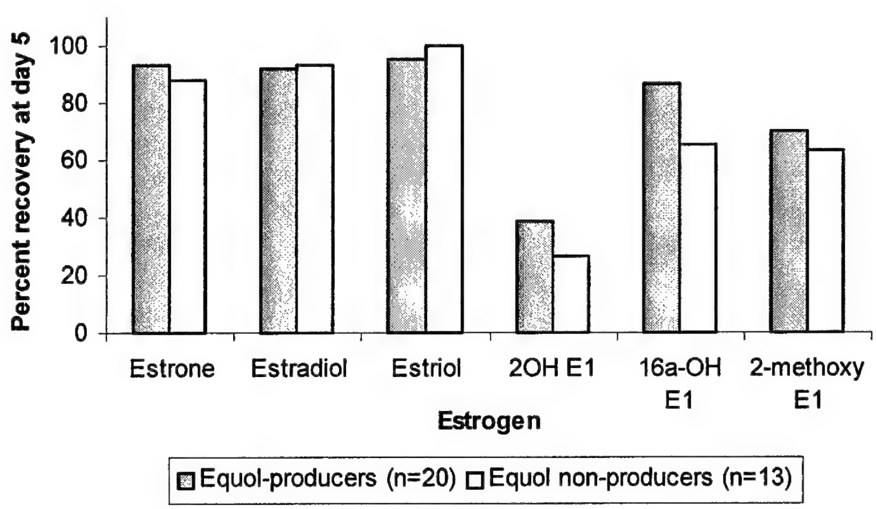
Appendix 2

Figure 2. Percentage recoveries of estrogens from control tubes at day zero and after 5 days at 37°C (panel A) and percentage recoveries of estrogens and their metabolites after a five-day incubation with feces, stratified by equol-producer status (panel B)

A



B



Appendix 3

Table 1. Summary of the *in vitro* metabolism of estrogens by fecal bacteria from equol-producers (EP) and equol non-producers (ENP) [a]

Estrogen	Metabolite	EP n (%) that converted (CONV.)	ENP n (%) that converted (CONV.)	χ^2 *p* value	EP % conversion CONV. only (range) [b]	ENP % conversion CONV. only (range) [c]	*p* CONV.	EP % conversion ALL (range)	ENP % conversion ALL (range)	*p* ALL
E1	E2	20 (100)	13 (100)	-	68 (41-90)	57 (25-82)	0.05	68 (41-90)	57 (25-82)	0.05
E2	E1	14 (70)	9 (69)	0.96	15 (8-24)	16 (7-25)	0.46	10 (0-24)	11 (0-25)	0.67
2-OH E1	2-OH E2	19 (95)	9 (69)	0.04	36 (13-108) [d]	31 (17-58)	0.55	32 (0-108)	22 (0-58)	0.21
2-OH E1	2-methoxy E2	2 (10)	0	0.24	11 (9-14)	0	- [e]	1 (0-9)	0	-
16α-OH E1	E3	18 (90)	9 (69)	0.13	84 (5-106)	62 (22-97)	0.03	75 (0-106)	43 (0-97)	0.02
16α-OH E1	17-epi E3	5 (25)	5 (38)	0.41	17 (11-24)	33 (8-108)	0.41	4 (0-24)	12 (0-108)	0.24
16α-OH E1	2-OH E1	3 (20)	2 (15)	0.98	14 (9-20)	14 (11-16)	-	2 (0-20)	2 (0-14)	-
16α-OH E1	16-epi E3	2 (10)	1 (8)	0.82	15 (13-16)	19 (n/a) [f]	-	1 (0-16)	1 (0-19)	-
16α-OH E1	16-keto E2	0	1 (8)	0.21	0	6 (n/a)	-	0	0.3 (0-4)	-
2-methoxy E1	2-methoxy E2	13 (72)	7 (54)	0.29	58 (19-93)	56 (12-87)	0.90	39 (0-93)	30 (0-87)	0.47
2-methoxy E1	2-OH E2	8 (44)	9 (69)	0.17	48 (35-60)	43 (15-87)	0.63	18 (0-52)	28 (0-87)	0.27
2-methoxy E1	2-OH E1	4 (22)	2 (15)	0.63	26 (6-43)	20 (19-21)	-	4 (0-28)	2 (0-19)	-
2-methoxy E1	E2	1 (6)	0	0.39	11 (n/a)	0	-	0.2 (0-3.5)	0	-

[a] Data for the metabolism of 2-methoxy E1 available for 18 equol-producers; [b] EP: average of two tubes used for the following: E2 to E1 (n=1), 2-OH E1 to 2-OH E2 (n=1), 16α-OH E1 to 17-epi E3 (n=1), 16α-OH E1 to 2-OH E1 (n=2), 2-methoxy E1 to 2-methoxy E2 (n=2), 2-methoxy E1 to 2-OH E2 (n=3), 2-methoxy E1 to 2-OH E1 (n=1), and data from one tube only used for the following: 2-OH E1 to 2-OH E2 (n=2), 2-OH E1 to 2-methoxy E2 (n=1), 16α-OH E1 to E3 (n=1), 2-methoxy E1 to 2-methoxy E2 (n=1), 2-methoxy E1 to 2-OH E1 (n=1), 2-methoxy E1 to E2 (n=1); [c] ENP: average of two tubes used for the following: 16α-OH E1 to 17-epi E3 (n=2), 16α-OH E1 to 16-keto E2 (n=1), 2-methoxy E1 to 2-OH E2 (n=2), and data from one tube only used for the following: 2-methoxy E1 to 2-methoxy E2 (n=1), 2-methoxy E1 to 2-OH E1 (n=1); [d] range may be greater than 100% due to methodological variability in quantification; [e] t-test not done due to small numbers; [f] n/a = range not applicable because fecal bacteria from only one individual resulted in production of that metabolite.

Appendix 4 – DRAFT MANUSCRIPT – DO NOT CITE

DRAFT MANUSCRIPT

The *in vitro* metabolism of estrogens by fecal bacteria from equol-producers and non-producers

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Running title: Bacterial estrogen metabolism by equol-producers and non-producers

Appendix 4 – DRAFT MANUSCRIPT – DO NOT CITE

Introduction

The clearest risk factors for breast cancer are those associated with hormonal and reproductive factors that result in greater exposure to estrogens (1). Other hormones, including progesterone, prolactin, and testosterone also may be important risk factors for breast cancer (2), although the evidence to date is somewhat limited. The principal circulating estrogens in humans are estriol, estradiol, and estrone. Estradiol has received considerable attention as a risk factor for breast cancer <add references>, and it has been suggested that estradiol likely increases the risk of breast cancer through its proliferative actions (2).

The metabolism of endogenous and exogenous estrogens could impact the levels and types of estrogens that circulate in the body. There are numerous steps involved in estrogen metabolism, including liver metabolism, biliary secretion, and intestinal bacterial metabolism and reabsorption (3). Estrogens are absorbed and transported to the liver where they are conjugated primarily to glucuronic acid. These conjugates are excreted in bile and enter the gastrointestinal tract. Intestinal bacteria hydrolyze the conjugates, which enables the unbound estrogen to be reabsorbed, thus undergoing enterohepatic recirculation. In addition, the intestinal bacteria also are capable of specific oxidation and reduction reactions to produce various metabolites. Several species of bacteria are involved ion estrogen metabolism, and some data suggest that certain reactions may be species, or even strain, specific (4). Perturbations in intestinal bacterial populations, such as after the administration of antibiotics, can result in changes in fecal and urinary excretion and plasma concentrations of several estrogens (5). Thus, it is possible that, through effects on the metabolism and subsequent exposure to estrogens, inter-individual differences in colonic bacterial populations may contribute to variation in disease susceptibility.

Appendix 4 – DRAFT MANUSCRIPT – DO NOT CITE

The equol-producer and O-desmethylangolensin (ODMA)-producer phenotypes are markers of inter-individual differences in gut bacterial populations; daidzein, a soy isoflavone, is metabolized by colonic microflora to equol and / or ODMA, which can be measured in blood and urine. Studies show that only 30-50% of the population harbors equol-producing bacteria (6), and that approximately 80-90% of the population harbors ODMA-producing bacteria (ref). To date, the specific bacterium or bacteria responsible for these conversions have not been identified, therefore equol and / or ODMA production is essentially a marker of a particular, although yet-to-be-defined, colonic bacterial profile.

Several studies provide evidence for a potential relationship between equol and breast cancer risk. For example, in a case-control study among Australian women there was a significant trend towards a lower risk of breast cancer through increasing quartiles of equol excretion; risk for the highest quartile of equol excretion was one quarter that of the lowest quartile (7). Furthermore, in a soy supplementation study, premenopausal equol-producers had circulating levels of hormones more likely to be associated with a reduced risk of breast cancer, irrespective of isoflavone dose; specifically, in comparison with women who were equol non-producers, equol-producers had lower circulating concentrations of steroid hormones such as estrone, testosterone, androstenedione, and cortisol, and higher concentrations of sex hormone binding globulin (SHBG) and mid-luteal phase progesterone (8). If these differences are consistent over a woman's lifetime, exposure to steroid hormones could be substantially different by equol-producer phenotype. Furthermore, because the findings were irrespective of isoflavone dose, inherent differences in hormone metabolism may exist between equol-producers and non-producers. We hypothesize that the colonic bacteria responsible for, or associated with, the

equol-producer phenotype are involved in steroid hormone metabolism, and contribute to differences in hormone profiles in women.

The aim of this study is to determine, for the first time, whether the *in vitro* metabolism of estrogens by human fecal bacteria differs according to equol- or ODMA- producer phenotype.

Materials and Methods

Study participants

Participants were recruited from the Seattle, Washington area. Exclusion criteria included use of antibiotics in the three months prior to the study, and age less than 18 years. 115 participants (88 female and 27 male) were phenotyped for their equol-producer status using a soy challenge (see (9, 10) for more details). Based on their equol-producer status, 48 participants were asked to provide a fresh fecal sample on one occasion. Of these, 34 were from female participants; 20 were equol-producers, 12 were equol non-producers, and 2 had trace amounts of equol in their urine. Samples from these women were used in the present study. Fecal samples were frozen and stored as described previously (11). Institutional Review Boards of the Fred Hutchinson Cancer Research Center, Seattle, WA, and Bastyr University, Kenmore, WA, approved all study procedures, and all participants provided written informed consent.

In vitro incubations

In vitro experiments were carried out as described previously (11), with minor modifications. Thawed feces (approximately 1-2 ml of feces in a 10% glycerol solution) was added to 5 ml BHI media and inverted several times to mix. 20 μl of this bacterial broth was added to 1 ml BHI media containing 14 μg of an estrogen. The following estrogens were

included in the incubations: estrone, estradiol, estriol (in 16 incubation runs; 8 equol-producers and 8 non-producers), 2-hydroxyestrone, 16α-hydroxyestrone, and 2-methoxyestrone (Steraloids Inc. Newport, RI). Each estrogen was tested separately. In addition, 100 μl of the bacterial broth was added to 5 ml BHI media containing daidzein (Indofine Chemical Co, Somerville, NJ) at a concentration of 39.4 μmol/l. All estrogen incubations were carried out in triplicate, and daidzein incubations in duplicate. Tubes were incubated under anaerobic conditions at 37°C for 5 days using the Gas Pak jar system (Becton Dickinson, Sparks, MD). To monitor for losses over the 5-day incubation, two additional sets of tubes were prepared without feces; one set (one 1 ml tube per estrogen) was frozen on day 0, and the other set was included in the 5-day incubation. In all runs, a tube containing media only was incubated (to verify that the media had not become contaminated during preparation), and in the majority of runs, a tube containing daidzein without feces also was incubated (to monitor for losses of daidzein). After the 5-day incubation, all tubes containing fecal inoculate showed visible signs of bacterial growth.

Equol-producer status associated with each fecal sample had been determined prior to this study using a three-day soy challenge (10, 12). Fecal samples from 34 individuals were used; 20 were from equol-producers, 12 were from equol non-producers, and 2 were from individuals who had trace amounts of equol in their urine sample (i.e., where a definitive classification of equol-producer status could not be made based on their urine sample). Fecal samples were incubated for 5 days at 37°C with the following estrogens: estrone (E1), estradiol (E2), estriol (E3), 2-hydroxyestrone (2-OH E1), 16α-hydroxyestrone (16α-OH E1), and 2-methoxyestrone (2-methoxy E1). E3 was removed from the target list after 16 incubations (8 equol-producers and 8 non-producers) because there was no metabolism of E3 by fecal bacteria; others (4) have reported similar findings. The following estrogens were included in the calibration curves for

quantifying estrogens and their metabolites after the 5-day incubation: E1, E2, E3, 2-OH E1, 16α-OH E1, 2-methoxy E1, 16-keto E2, 16-epi E3, 17α-E2, 2-OH E2, 2-methoxy E2, and 17-epi E3.

Extraction and quantification of estrogens and isoflavones

Estrogens and isoflavones were extracted from the BHI media using HPLC grade ethyl acetate <add supplier name and State>. For samples with a total volume of 1 ml, the entire sample was used, and for samples with a total volume of 5 ml, tubes were centrifuged and 1.0 ml removed into a clean test tube for extraction. An internal standard (100 µl of a 50 ng/µl solution of enterodiol in methanol) was added to each sample, and then 2 ml ethyl acetate was added. Tubes were vortexed and then centrifuged for 20 minutes at 3200 rpm. The ethyl acetate layer was then removed using a glass pipette and placed in a clean test tube. This was repeated twice more, and the 3 ethyl acetate layers were combined in one tube. Ethyl acetate was evaporated under nitrogen at 37°C, and samples were either derivatized immediately (see below) or re-hydrated in 0.5 ml methanol for storage at -20°C. Prior to derivatization, stored samples were brought to room temperature and the methanol was evaporated under nitrogen. For derivatization, samples were baked at 105°C for 20 minutes to ensure the removal of all water. Samples were cooled to room temperature then 200 µl of 15 % MSTFA in acetonitrile (Pierce Chemical Co., <State>) was added to each sample. Samples were incubated for 30 minutes at room temperature in a vacuum dessicator, and then analyzed for estrogens and isoflavones by isotope dilution gas chromatography-mass spectrometry in the SIM mode (HP 6890 GC, HP 5973 MSD). The column used was a DB-17MS capillary (122-4732, J &W Scientific), 30 m x 0.25 mm x 0.25 µm film thickness. Helium was the carrier gas with a flow rate of 1 ml / min.

The column temperature was as follows: 100°C for 1 minute, ramp at 17°C / min to 250°C, no hold, then ramp at 2°C / min to a final temperature of 300°C, and hold for 1 min. The inlet temperature was held constant at 260°C and operated in a pulsed, splitless mode. The ion source and interface temperatures were 200°C and 250°C, respectively. The run time was 36 minutes, and the injection volume was 1 μl. The following estrogens were quantified: estrone (E1), 16-keto-estradiol, 17α-estradiol, 17β-estradiol, estriol, 16-epiestriol, 17-epiestriol, 2-hydroxyestrone, 2-hydroxyestradiol, 16α-hydroxyestrone, 2-methoxyestrone, and 2-methoxyestradiol. To verify equol producer status and to assess general patterns of daidzein metabolism *in vitro*, ODMA, daidzein, equol, and dihydrodaidzein were identified and semi-quantified in the same GC-MS run. Exact quantification was not possible because this method was not optimized for isoflavone analysis. However, the data are informative in terms of confirming the presence / absence of equol-producing bacteria in each incubation. All standards and quality control samples were prepared in 1 ml of BHI media and extracted and analyzed in the same manner as the samples. <add data on CVs>

Data analysis

Some sets of triplicates showed conversion to a metabolite in all tubes, in two tubes, or in one tube only. Chi square analysis was used to determine differences between equol-producers and non-producers in the proportion of samples that showed conversion to a particular metabolite; if any of the tubes in the triplicate showed conversion to a particular metabolite, it was classed as a converter. To determine differences between equol-producers and non-producers in the percent conversion of an estrogen to its metabolite, it was assessed in two ways: a) using data from only the tubes that showed conversion i.e., if two out of the three tubes

produced a specific metabolite, the mean of the two tubes was taken, and if only one out of the three tubes produced a specific metabolite, the percent conversion was based on data from that one tube; b) using data from all tubes i.e., averages were calculated for all tubes irrespective of whether or not conversion had occurred in all tubes. $P \leq 0.05$ was considered statistically significant.

Results

Data for one individual, who had been classed as an equol-producer using their urine sample, was not included in the analyses because their fecal bacteria did not convert daidzein to equol *in vitro*. Of the two individuals with trace amounts of equol in their urine sample, fecal bacteria from one individual produced equol, and fecal bacteria from the other did not metabolize daidzein. Thus, the findings are based on data from 20 equol-producers and 13 equol non-producers.

Daidzein was metabolized, *in vitro*, by fecal bacteria to equol, O-desmethylangolensin (O-DMA), and dihydrodaidzein (DHD). Fecal samples from 19 individuals who had been classed as equol-producers according to their urine sample and 1 individual who had trace amounts of equol in their urine sample, produced equol *in vitro*; of these, 15 produced equol only and 5 produced equol and DHD. Fecal samples from 12 individuals who had been classed as equol non-producers according to their urine sample and 1 individual who had trace amounts of equol in their urine sample, did not produce equol *in vitro*; of these, 4 did not metabolize daidzein, 4 produced DHD only, 3 produced O-DMA only, and, 2 produced DHD and O-DMA.

Recoveries of target estrogens from control samples from day 0, with and without feces, and from control tubes that had been incubated at 37°C for 5 days, were excellent (≥93%) with

the exception of 2-OH E1; recoveries of 2-OH E1 from control tubes averaged approximately 30% [appendix 2 (figure 2), panel A]. Recoveries of estrogens and their metabolites after a 5-day incubation with feces were excellent for E1, E2, and E3 (≥88%). Recovery of 2-OH E1 plus its metabolites was highly variable for incubated samples, and averaged less than 40% following the 5-day incubation [appendix 2 (figure 2), panel B]. Other investigators have reported similar problems (13). Recoveries of 16α-OH E1 and its metabolites from samples incubated with feces averaged 87% and 65% for equol-producers and non-producers, respectively, and recoveries of 2-methoxy E1 and its metabolites averaged 70% and 63% for equol-producers and non-producers, respectively [appendix 2 (figure 2), panel B]. These recoveries are lower than were observed with control tubes (≥93% and ≥98% for 16α-OH E1 and 2-methoxy E1, respectively).

Large inter-individual variation in the metabolism of estrogens was apparent, as evidenced by the range of percent conversions shown in Table 1. Several reduction, oxidation, and demethylation reactions were observed among equol-producers and non-producers. A summary of the *in vitro* metabolism of estrogens by fecal bacteria from equol-producers and non-producers is shown in Table 1. Compared to equol non-producers, equol-producers converted significantly more E1 to E2 ($p=0.05$), and significantly more 16α-OH E1 to E3 ($p=0.03$). All other differences between equol-producers and non-producers were not statistically significant ($p>0.05$). Some metabolites were produced only by equol-producers (i.e., the production of 2-methoxy E2 from 2-OH E1 and E2 from 2-methoxy E1) or by equol non-producers (i.e., the production of 16-keto E2 from 16α-OH E1).

Discussion

There was considerable inter-individual variation in the *in vitro* metabolism of estrogens by human fecal bacteria, and some statistically significant differences between equol-producers and non-producers for the metabolism of some estrogens.

Similar to other studies (4) human fecal bacteria did not metabolize estriol. A greater proportion of equol-producers than non-producers converted 2-OH E1 to 2-OH E2, but low overall recoveries of 2-OH E1 make it difficult to draw any conclusions regarding the intestinal bacterial metabolism of 2-OH E1. On average, equol producers, compared with non-producers, converted more 16α-OH E1 to E3 and more E1 to E2.

Acknowledgements

The authors wish to thank all study participants.

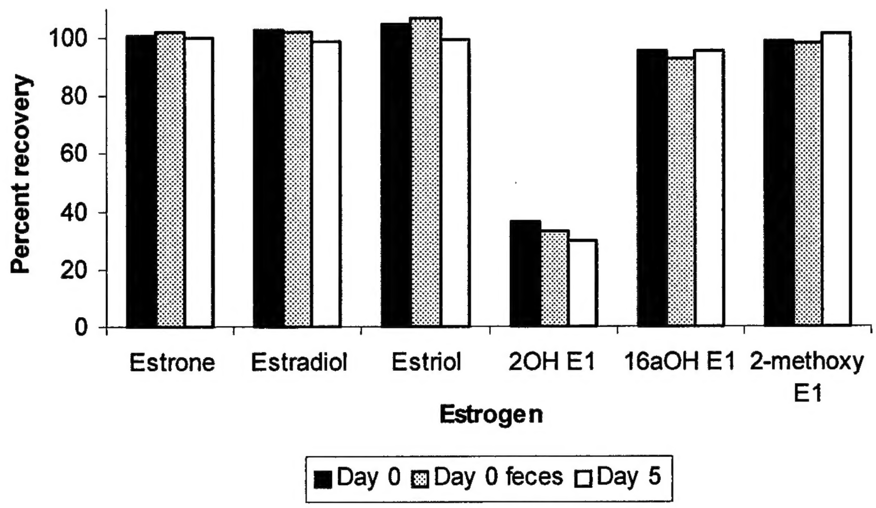
Table 1. Summary of the *in vitro* metabolism of estrogens by fecal bacteria from equol-producers (EP) and equol non-producers (ENP) [a]

Estrogen	Metabolite	EP n (%) that converted (CONV.)	ENP n (%) that converted (CONV.)	χ^2 p value	EP % conversion CONV. only (range) [b]	ENP % conversion CONV. only (range) [c]	p CONV.	EP % conversion ALL (range)	ENP % conversion ALL (range)	p ALL
E1	E2	20 (100)	13 (100)	-	68 (41-90)	57 (25-82)	0.05	68 (41-90)	57 (25-82)	0.05
E2	E1	14 (70)	9 (69)	0.96	15 (8-24)	16 (7-25)	0.46	10 (0-24)	11 (0-25)	0.67
2-OH E1	2-OH E2	19 (95)	9 (69)	0.04	36 (13-108) [d]	31 (17-58)	0.55	32 (0-108)	22 (0-58)	0.21
2-OH E1	2-methoxy E2	2 (10)	0	0.24	11 (9-14)	0	- [e]	1 (0-9)	0	-
16α-OH E1	E3	18 (90)	9 (69)	0.13	84 (5-106)	62 (22-97)	0.03	75 (0-106)	43 (0-97)	0.02
16α-OH E1	17-epi E3	5 (25)	5 (38)	0.41	17 (11-24)	33 (8-108)	0.41	4 (0-24)	12 (0-108)	0.24
16α-OH E1	2-OH E1	3 (20)	2 (15)	0.98	14 (9-20)	14 (11-16)	-	2 (0-20)	2 (0-14)	-
16α-OH E1	16-epi E3	2 (10)	1 (8)	0.82	15 (13-16)	19 (n/a) [f]	-	1 (0-16)	1 (0-19)	-
16α-OH E1	16-keto E2	0	1 (8)	0.21	0	6 (n/a)	-	0	0.3 (0-4)	-
2-methoxy E1	2-methoxy E2	13 (72)	7 (54)	0.29	58 (19-93)	56 (12-87)	0.90	39 (0-93)	30 (0-87)	0.47
2-methoxy E1	2-OH E2	8 (44)	9 (69)	0.17	48 (35-60)	43 (15-87)	0.63	18 (0-52)	28 (0-87)	0.27
2-methoxy E1	2-OH E1	4 (22)	2 (15)	0.63	26 (6-43)	20 (19-21)	-	4 (0-28)	2 (0-19)	-
2-methoxy E1	E2	1 (6)	0	0.39	11 (n/a)	0	-	0.2 (0-3.5)	0	-

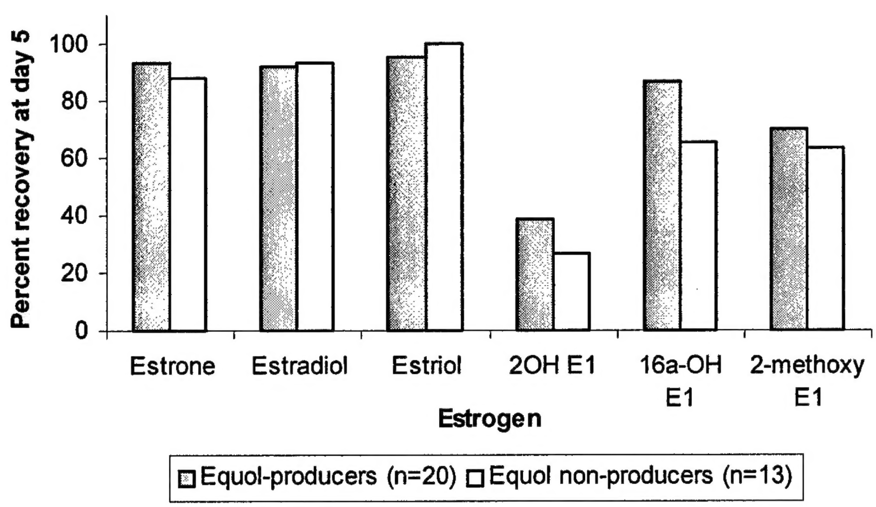
[a] Data for the metabolism of 2-methoxy E1 available for 18 equol-producers; [b] EP: average of two tubes used for the following: E2 to E1 (n=1), 2-OH E1 to 2-OH E2 (n=1), 16α-OH E1 to 17-epi E3 (n=1), 16α-OH E1 to 2-OH E1 (n=2), 2-methoxy E1 to 2-methoxy E2 (n=2), 2-methoxy E1 to 2-OH E2 (n=3), 2-methoxy E1 to 2-OH E1 (n=1), and data from one tube only used for the following: 2-OH E1 to 2-OH E2 (n=2), 2-OH E1 to 2-methoxy E2 (n=1), 16α-OH E1 to E3 (n=1), 2-methoxy E1 to 2-methoxy E2 (n=1), 2-methoxy E1 to 2-OH E1 (n=1), 2-methoxy E1 to E2 (n=1); [c] ENP: average of two tubes used for the following: 16α-OH E1 to 17-epi E3 (n=2), 16α-OH E1 to 16-keto E2 (n=1), 2-methoxy E1 to 2-OH E2 (n=2), and data from one tube only used for the following: 2-methoxy E1 to 2-methoxy E2 (n=1), 2-methoxy E1 to 2-OH E1 (n=1); [d] range may be greater than 100% due to methodological variability in quantification; [e] t-test not done due to small numbers; [f] n/a = range not applicable because fecal bacteria from only one individual resulted in production of that metabolite.

Figure 1. Percentage recoveries of estrogens from control tubes at day zero and after 5 days at 37°C (panel A) and percentage recoveries of estrogens and their metabolites after a five-day incubation with feces, stratified by equol-producer status (panel B)

A



B



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